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Elimination of Matrix-Based Interferences to a Fluorescent Nitrite/Nitrate Assay by a Simple Filtration Procedure[1]

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Pathophysiological levels of oxygen radical metabolites have been studied as indicators of trauma caused by burn insult. The 2,3-diaminonaphthalene assay is routinely used in the determination of nitrite/nitrate levels in biological fluids and cellular extracts as one indicator of nitric oxide activity. Several laboratories, including ours, have noted matrix-based interferences resulting in decreased assay sensitivity during nitrite/nitrate analysis. We evaluated filtration using Millipore Ultrafree-MC 10,000 NMWL filters for the ability to eliminate matrix-based interferences from human serum and tissue culture medium, thereby restoring assay sensitivity.

The importance of nitric oxide (NO)[3] as a mediator in many molecular events is underscored by its role in physiological processes such as vasodilation, sepsis, thrombosis, immune function, and neurotransmission (1, 2). NO is biosynthesized from the amino acid L-arginine by the action of nitric oxide synthase (NOS). The resulting NO free radical species may quickly react with molecular oxygen and water to produce nitrites and nitrates.

A number of methods are available for the detection of NO in biological extracts. The Griess (3) reaction is widely used because of its simplicity and sensitive detection limits at about 100 nM. This colorimetric assay consists of the reaction of nitrite/nitrates with the Griess reagent to form a purple azo derivative that can be monitored at 549 nm using spectrophotometry. Excess NADPH, which interferes with the Griess assay, can be removed by pretreatment with glucose-6-phosphate dehydrogenase. Gas chromatographic analysis of NO (4) has been reported, but it requires extensive sample preparation procedures and expensive analytical equipment that is not commonly available in many laboratories, and it suffers from poor reproducibility. Methodology is also available for the detection of NO using high-performance liquid chromatography (5, 6), capillary electrophoresis (7), fluorometry (4, 8), and chemiluminescence (4, 9).

One method for detection of NO uses the reagent 2,3-diaminonaphthalene (DAN). The assay, as reported by Misko *et al.* (1), uses the reaction of DAN with NO_2^- under acidic conditions to form a detectable fluorescent naphthotriazole (Fig. 1). The reaction proceeds at acidic pH at room temperature. Fluorescence (excitation 330 nm, emission 426 nm) is monitored following the addition of NaOH, which raises pH, resulting in lower background and increased sensitivity (10). Detection limits of NO achieved by this method are in the high-pM range.

Nitrite/nitrate analysis in biological/cellular extracts and body fluids is important in the study of nitric oxide metabolism. Pathophysiological levels of these metabolites correlate with the degree of trauma resulting from the hypermetabolic activity induced by burn injury. The assay is routinely used in our laboratory for

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[3] Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; DAN, 2,3-diaminonaphthalene; RFU, relative fluorescence unit; NHS, normal human serum; FBS, fetal bovine serum; BSA, bovine serum albumin.

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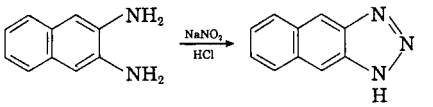

DAN(2,3-diaminonaphthalene) 1(*H*)-naphthotriazole

FIG. 1. DAN reaction.

the measurement of nitrite/nitrate levels in body fluids and biological and cellular extracts, from both human and animal subjects. We have observed interferences resulting in decreased assay sensitivity due to the nature of these samples. In this study, we evaluated a filtration procedure, routinely used in many laboratories, for its ability to restore assay sensitivity in relevant matrices.

MATERIALS AND METHODS

Nitrite/Nitrate Assay

2,3-Diaminonaphthalene (Chem Service, West Chester, PA), under acidic conditions, reacts with nitrite to form the fluorescent molecule 1*H*-naphthotriazole. The two matrices that were examined were normal human serum (Sigma, St. Louis, MO) and tissue culture medium with 10% fetal bovine serum (RPMI 1640; GIBCO, Grand Island, NY). One hundred microliters of the appropriate matrix was added to each well of a 96-well plate (Immunolon; Dynex Technologies, Inc., Chantilly, VA), containing 20 μl of NADPH/nitrate reductase (14 mU) (Sigma) and 20 μl of 50 mM Tris at pH 7.6, and incubated for 90 min at 25°C. Next, 10 μl of DAN solution (0.05 mg/ml in 0.62 M HCl) was added to each well and incubated for an additional 10 min. The reaction was stopped by the addition of 10 μl of 2.8 N NaOH. The fluorescence of the 1*H*-naphthotriazole was measured using a Perkin–Elmer LS 50B bioluminescence spectrophotometer (excitation 330 nm/emission 426 nm, slits 2.5 nm). Standard curves were prepared by plotting the mean relative fluorescence units (RFUs) of triplicate analysis as a function of nitrite concentration and fit to a straight line by linear regression analysis. Samples were assayed in triplicate on each assay. Each test was repeated a minimum of three times. Intra- and interassay coefficients of variation for high (1000 pM), medium (600 pM), and low (100 pM) quality control samples were calculated for each matrix.

Sample Filtration Procedure

The samples were filtered through a 10,000 MW cutoff Ultrafree-MC microcentrifuge filter (Millipore, Bedford, MA) by centrifugation at 300*g* for 30 min at 25°C. We observed that sample viscosity and the amount of protein aggregates affected the filtration time. In some cases, several filtration steps were required to obtain enough filtrate to perform the assay in triplicate. A colorless filtrate was obtained after serum filtration, while no color change was observed in tissue culture medium. Nitrite/nitrate analysis was performed the same day as filtration of samples.

Nitrite/Nitrate Assay in Normal Human Serum and Tissue Culture Medium

To determine assay interference by normal human serum (NHS) or tissue culture medium containing 10% fetal bovine serum (FBS), standard curves were constructed in respective matrices. Samples were spiked with a freshly prepared (100 μM) stock solution of sodium nitrite (Sigma) in distilled H_2O to give final concentrations of 0, 100, 200, 300, and 600 pM. Half of each spiked sample was filtered and the remainder

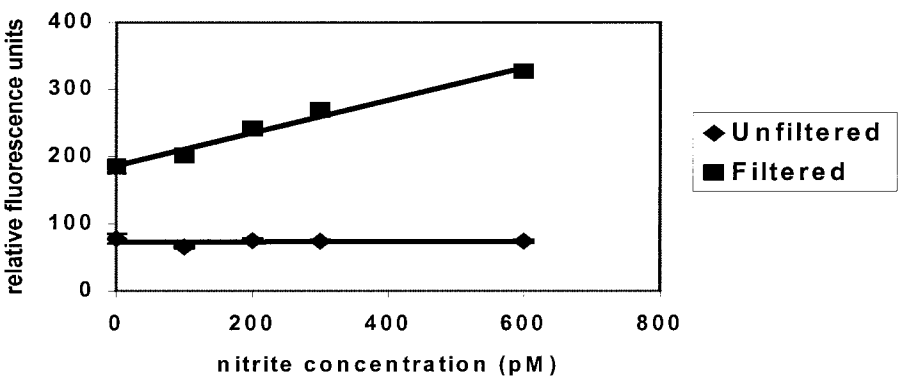

FIG. 2. Comparison of filtered and unfiltered nitrite/nitrate levels in normal serum. Filtration restored assay sensitivity in NHS samples. Linear standard curves were obtained for filtered serum (r^2 = 0.98) and compared to unfiltered (r^2 = 0.004) serum. Nonspecific assay fluorescence was not subtracted. There was a notable increase in slope from the unfiltered to filtered sample (0.0013 to 0.2432 RFU/pM, respectively).

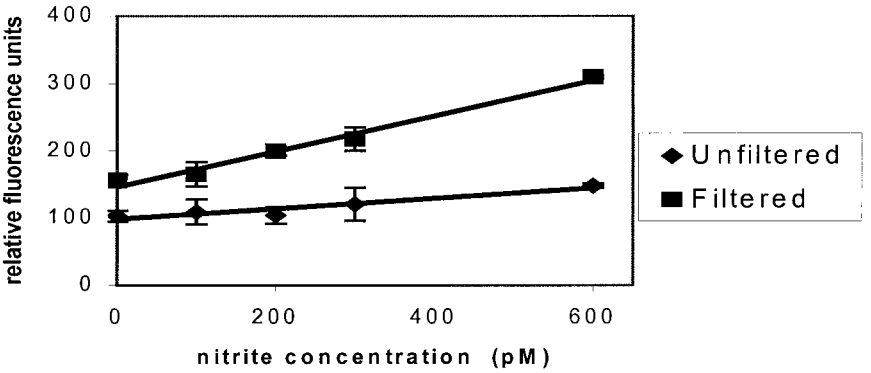

FIG. 3. Comparison of filtered and unfiltered nitrite/nitrate levels in medium with 10% FBS. FBS is a supplement commonly used in tissue culture medium. Filtration results in improved sensitivity as shown by an increased slope of the unfiltered (0.078 RFU/pM) to the filtered sample (0.265 RFU/pM). Nonspecific assay fluorescence was not subtracted.

saved without filtration. Slopes and intercepts of the resulting linear regression lines of these standard curves were used to evaluate the dynamic range of each assay condition. Each concentration was compared to the 0 pM concentration by *t* test to determine sensitivity.

Potential Source of Interference

To determine if decreased assay sensitivity was caused by nonspecific binding of nitrite/nitrate to serum albumin, a common binding protein, bovine serum albumin (BSA), was mixed with tissue culture medium at concentrations of 0, 0.5, 1.0, 3.0, and 5.0 mg/ml (w/v). The spiked nitrite concentration was 200 pM in all samples. Both filtered and unfiltered samples were analyzed for nitrites.

Statistical Analysis

Regression analysis was used to determine the relationship between relative fluorescence and actual nitrite concentration. Sensitivity was defined as the lowest nitrite concentration that could be statistically differentiated from the 0 pM concentration. This was determined by *t* test. Statistical analyses were done using SAS statistical software (11).

RESULTS AND DISCUSSION

Our results clearly show filtration restores assay sensitivity in undiluted NHS and tissue culture medium (RPMI 1640 containing 10% FBS). The filtration procedure was verified by preparing standard curves in the different matrices. Standard curves constructed in unfiltered samples resulted in slopes (NHS, 0.0013 RFU/pM; tissue culture medium, 0.078 RFU/pM) indicative of a lower dynamic range, as compared to the filtered samples (NHS, 0.2432 RFU/pM; tissue culture medium, 0.2644 RFU/pM). Standard curves were linear for filtered NHS ($r^2 = 0.980$) and tissue culture medium ($r^2 = 0.984$) over the range of 0–600 pM (Figs. 2 and 3, respectively). The detection limit of the standard curves in filtered matrices was in the 100–200 pM range. Sensitivity was 200 pM in filtered NHS, while the 600 pM concentration could not be differentiated from 0 pM when unfiltered NHS was assayed (Table 1). Filtration of serum resulted in a slope of 0.2432 RFU/pM, which was greater ($P < 0.01$) than the slope of 0.0013 RFU/pM for unfiltered serum (Fig. 2). These detection limits may be further improved by systematically optimizing reagent concentrations and adjusting incubation times. Recovery and nonspecific binding to the filters was not a problem in any of the assays. The percent recovery from the filters at 100, 200, 300, and 600 pM was >90%. The percent recovery at 1 mM was >97% (data not shown). Concentrations were not corrected for these losses. When tissue culture medium was filtered, similar results were obtained. Sensitivity was 100 pM after filtration compared to >1000 pM without filtration (Table 1). The slope for tissue culture medium was increased ($P < 0.01$) compared to unfiltered medium (0.2644 vs 0.078 RFU/pM, respectively) as shown in Fig. 3.

Table 2 shows the intra- and interassay coefficients of variation for the filtered and unfiltered samples. Intraassay coefficients of variation were no greater

TABLE 1

Minimum Level of Detection

Matrix	Treatment	Sensitivity (pM)	*P* Value
NHS	Filtered	200	<0.01
	Unfiltered	>1000	
FBS	Filtered	100	<0.05
	Unfiltered	>1000	

TABLE 2

Intra- and Interassay Variation

	Intraassay variation (%)			Interassay variation (%)		
	Low[a]	Medium[b]	High[c]	Low[a]	Medium[b]	High[c]
Serum						
Filtered	1	3	3	5	6	3
Unfiltered	2	4	3	10	5	3
FBS/Medium						
Filtered	5	5	3	13	19	15
Unfiltered	4	5	5	44	46	30

[a] 100 pM.
[b] 600 pM.
[c] 1000 pM.

than 5% for filtered and unfiltered samples in both normal human serum and tissue culture medium. Intraassay coefficients of variation for both filtered and unfiltered normal human serum were 10% or less. Interassay coefficients of variation for filtered tissue culture medium were no greater than 19% but were as high as 46% for unfiltered medium.

Macromolecules over 10,000 MW appear to be in part responsible for the interference of the DAN-based nitrite/nitrate assay. Nonspecific binding of the negatively charged NO could reduce subsequent availability of nitrite/nitrates for the DAN reaction. Nitric oxide, a highly reactive free radical species, can react with numerous molecules under physiological conditions. One important reaction is with O_2 and water to form nitrite/nitrates (detectable by the DAN assay). Nitric oxide can also react with proteins, such as hemoglobin, to form iron–nitrosyl compounds or other nitrosylated adducts (not detectable by the DAN assay) (1). Such reactions must be considered when interpreting results of this assay for analysis of NO. However, this is not a factor here because the source of nitrite used in our standard curves was nitrite salt, not the nitrite precursor NO. Figure 4 shows that albumin does not interfere with the DAN assay. If protein binding were the factor that reduced the sensitivity of the assay, then one would expect to see decreased fluorescence as the albumin concentration is increased due to nonspecific binding of the nitrite/nitrate to the albumin. A possible explanation for the filtered samples having higher signal could be the removal of macromolecules, such as flavins and flavoproteins, responsible for quenching. The simple filtration procedure described here clearly results in decreased quenching, restoring assay sensitivity.

Several laboratories have reported the use of the filters mentioned in this work as a sample preparation procedure before analyzing urine and to remove large molecular weight macromolecules, such as hemoglobin, from serum samples (1, 2). In this report, we

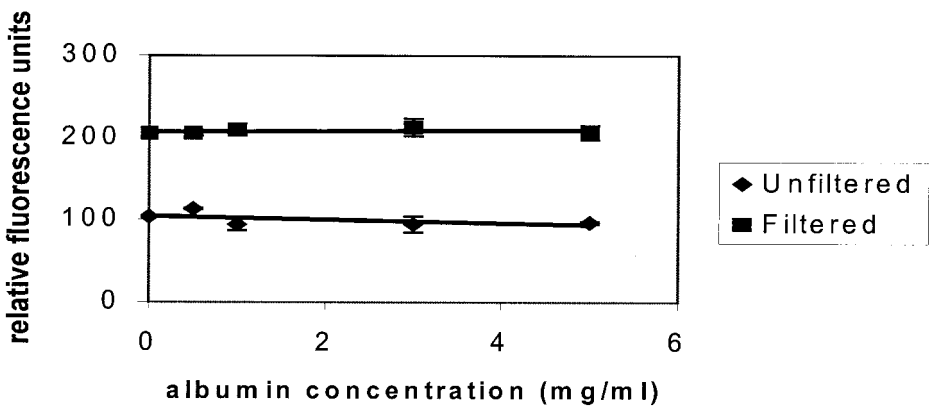

FIG. 4. Comparison of nitrite/nitrate level in medium with increased albumin concentrations. Albumin (BSA), a major component of serum, was added to a constant amount of nitrite (200 pM). Both filtered and unfiltered samples were analyzed for nitrites using the DAN reaction. The plotted line for the filtered samples shows the expected signal for 200 pM with increasing albumin concentration.

expanded those observations to include normal human serum and FBS-supplemented tissue culture medium. The filtration procedure as described in this work is a simple and cost-effective way to restore the sensitivity of the DAN assay for nitrite/nitrates in serum and FBS-supplemented tissue culture medium.

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